A CHEMICAL METHOD FOR THE DETERMINATION OF THIAMIN

BY

ERNST RUDOLF KIRCH

Ph. C., University of Illinois, 1928
B. S., Loyola University, 1931
M. S., University of Chicago, 1934

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
PHYSIOLOGICAL CHEMISTRY IN THE
GRADUATE SCHOOL OF THE
UNIVERSITY OF
ILLINOIS, 1941

CHICAGO, ILLINOIS
1941

A CHEMICAL METHOD FOR THE DETERMINATION OF THIAMIN

BY

ERNST RUDOLF KIRCH

Ph. C., University of Illinois, 1928
B. S., Loyola University, 1931
M. S., University of Chicago, 1934

AN ABSTRACT OF A THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
PHYSIOLOGICAL CHEMISTRY IN THE
GRADUATE SCHOOL OF THE
UNIVERSITY OF
ILLINOIS, 1941

CHICAGO, ILLINOIS

1941

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. Olaf Bergeim for his advice and assistance in the direction of this investigation.

A CHEMICAL METHOD FOR THE DETERMINATION OF THIAMIN

Advances in the study of the physiological behavior of thiamin make a direct chemical quantitative estimation of this vitamin highly desirable. Several chemical and biochemical tests have been developed which can be applied to solutions or eluates of the compound.

Of the chemical tests in use at the present time none can be applied to extracts of various materials without a preliminary process of purification, which give rise to large losses of the vitamin. Some of these tests are not even specific. Whereas most of the biochemical procedures give accurate results and are ideally suited for microdeterminations, they are too time consuming.

A diazo-reaction, which would produce with thiamin a precipitate or a specific color in an aqueous solution which was extractable with a selective solvent would be desirable. The elimination of the adsorption process and other means of purification would greatly improve a chemical assay for thiamin. It is believed that the search for such a reagent has been successful to the extent that the test developed can be applied quantitatively to solutions containing 1 to 2 units (0.3 to 0.6 microgm.) of thiamin per 100 in a variety of biological materials.

Directions for the Test: 5 cc. of a diazo solution, consisting of equal parts of a 0.5% alcoholic solution of trichloroacetate of ethyl p-aminobenzoate and a 0.5% solution of sodium nitrite

in 85% alcohol, are added to the solution of vitamin B_1 to be tested. The pH of the reaction mixture should be about 5.0. The addition of the diazo solution is followed after about 2 minutes with 1 N sodium hydroxide solution until a pH of 7.5-8.0 has been reached. A pink to red color will develop varying in intensity depending upon the concentration of thiamin. This color is then extracted with 5 cc. iso-amyl alcohol.

The degree of accuracy using standard solutions of varying concentrations of thiamin is ascertained. A Pulfrich photometer for measuring the color was used; a standard curve is then constructed on semi-logarithmic graph paper from which the concentration of the vitamin could be read directly.

Table I gives results showing that as concentrations of thiamin increase from 2 to 30 units per 5 cc. of solution the recoveries are quantitative.

TABLE I.

Reproducibility of Results Using the Method for the Determination of Thiamin.

Units 5 cc.	Number of Determinations	Variation in Deviations of the Average	
		Units B_1	Units B_1
1	20	1	1
2	20	2	2
5	20	5	5
10	20	9	10.5
15	20	14	16
20	20	19	21
30	20	28	32

Numerous biologically important compounds were tested with the reagent for any possible interference. Of those investigated, adrenalin gave a distinct red color which, unlike that produced by thiamin, was unstable and disappeared within 8-10 hours. Resorcin, phloroglucin and phenol in a more concentrated solution gave an orange-brown color fading after 14 hours. The color produced by thiamin has been found to remain stable for at least 3 months.

The thiazole part of the thiamin molecule seems to be the one concerned with the color formation since some of the pyrimidine intermediates did not give any color with the reagent, whereas 4-methyl-5-beta hydroxy ethyl thiazole did give a pink to red color. The intensity of the color produced was far less than with thiamin since 100 times the amount of the thiazole derivative had to be used to produce a color of the same intensity as produced by a certain amount of the thiamin.

Of the various other vitamins, ascorbic acid was found to prevent the color formation. This was expected because of the reducing properties of vitamin C. If solutions to be tested for vitamin B_1 also contain vitamin C, the interference due to the latter can be overcome by destroying this substance with dilute potassium permanganate before testing for the thiamin.

II.

Vitamin B_1 exists in nature as the pyrophosphate to constitute cocarboxylase, the coenzyme of carboxylase, which has a biological activity corresponding to that of thiamin.

Tests conducted on fresh solutions of pure cocarboxylase showed that this compound did not react with diazotized ethyl p-aminobezoate.

In order to estimate the total thiamin content of yeast it is necessary to hydrolyze quantitatively the cocarboxylase to the free vitamin. Acid hydrolysis converts the ester to the monophosphate only. Alkaline treatment destroys the whole thiamin molecule.

The use of the enzyme phosphatase present in yeast has been resorted to. It has been found that this enzyme in a 30% ethyl alcohol suspension of yeast at a temperature of 90 – 95° C. and a pH range of 4.0 – 7.0 acts quantitively and in a relatively short time in splitting the ester to the free vitamin.

Yeast suspensions in water or 30% ethyl alcohol were heated for 3 minutes in boiling water, filtered and the filtrate tested for thiamin. Yeast treated in this manner was found to contain about 3 to 4 times as much vitamin B_1 as the yeast treated with sand and extracted with cold water.
Table II will illustrate this point.

TABLE II.

2 gm. of yeast were suspended in 20 cc. of liquid; the test was carried out on 10 cc. of the filtrate representing 1 gm. of yeast. The samples were treated as follows:

1. Boiled in 30% alcohol for 2 minutes
2. Boiled in water for 3 minutes
3. Yeast rubbed with sand and then suspended in 30% alcohol
4. Yeast treated as in No. 3 but using 30% alcohol containing 6 units of cocarboxylase per 10 cc.
5. Yeast rubbed with sand and suspended in water containing 6 units of cocarboxylase per 10 cc.

Sample	Units B_1/gm.	Units B_1/Cake
1	15	187.5
2	15	187.5
3	15	187.5
4	21	---
5	4	50.0

III.

Early quantitative estimations of thiamin in urine were carried out by the use of the bradycardia method of Birch and Harris. This procedure requires the maintenance of a rat colony as well as 7 – 10 days for a determination.

The use of the thiochrome method of Jansen as well as the method of Melnick and Field requires the preliminary isolation of the thiamin by the use of adsorbents.

The test reported in Part I has been modified to apply to urine directly. The urine is concentrated in vacuo to 1/10 of its original volume. Aliquot portions of this concentrate are tested for thiamin directly. Since ascorbic acid is also present in the urine, the former is oxidized with 1% potassium permanganate solution prior to the addition of the diazo solution. Results obtained indicate a 98% recovery of the thiamin added.

Since the urine of patients may contain various drugs used for medication, a number of these have been added to urine of known thiamin concentration and tested for any possible interference. Sulfanilamide, sulfthiazole, adrenalin, phenol, methenamine, atropine, aspirin, and histamin were some of the compounds tested. Adrenalin was the only one to react to give a red color which faded completely within 12 hours.

The stability of thiamin in the bladder has been studied. Results indicate that there is no appreciable destruction of thiamin in urine in vivo of the physiological pH range.

The vitamin is excreted in the urine as free thiamin and not in its phosphorylated form.

Cocarboxylase added to urine did not show any increase in the thiamin content in the urine, thus indicating the absence of phosphatase in the latter. Like wise the addition of phosphatase to the urine did not show any increase in the free vitamin in the urine, showing the absence of cocarboxylase in the urine. Results thus obtained indicates that dephosphorylation of the thiamin takes place in the organism itself and not after the formation of the urine.

Determination of vitamin B_1 excreted in the urine for the recognition of deficiency is only approximately suitable since the quantity of the excreted thiamin depends upon the diet, exercise and various other conditions. Normally the excretion fluctuates within a wide range. Variations from as low as 4% to as high as 50% of thiamin administered orally have been found in the urine. Fecal excretion, however, is rather low and constant. These results indicate therefore that a large part of the vitamin ingested is presumably destroyed by the metabolic changes in the tissues.

SUMMARY

I

1. A chemical method for the determination of thiamin been described in which diazaotized ethyl p-aminobenzoate reacts with the vitamin to form a pink-red compound which can be extracted quantitatively with isoamyl alcohol.

2. A modification of the procedure to eliminate the interference of ascorbic acid has been developed.

3. The method is specific for free thiamin.

II

1. By the method based upon the reaction of diazotized ethyl p-aminobenzoate and thiamin it is not possible to determine the vitamin in the phosphorylated form.

2. By use of the enzyme phosphatase present in yeast it is possible to hydrolyze the cocarboxylase to free thiamin in a relatively short time.

3. The procedure has been applied to the estimation of thiamin in yeast and the latter was found to contain about 75% of its thiamin in the phosphorylated form.

III

1. The chemical test based on the reaction of thiamin and diazotized ethyl p-aminobenzoate has been successfully applied to the estimation of the vitamin in urine.

2. Variations in urinary pH in vivo do not have any destructive effects on the thiamin.

3. The thiamin is excreted in the urine as the free vitamin and not in its phosphorylated form.

4. Since the urinary excretion of thiamin fluctuates over a wide range, the determination of the vitamin excreted in the urine is only approximately suitable for the recognition of any vitamin B_1 deficiency.

VITA

Ernst Rudolf Kirch was born in Kirn, Germany, June 24, 1906. Received elementary education in Daun, Germany and attended the Gymnasium at Rheinbach, Germany. Received Ph. C. from the University of Illinois in 1928: Received B. S. from Loyola University, Chicago, in 1931: Received M. S. from the University of Chicago in 1934: Assistant in Chemistry, University of Illinois College of Pharmacy from 1928 to 1939: Instructor in Chemistry, University of Illinois College of Pharmacy, 1939 — to date. Member of Sigma Xi, Rho Chi and Kappa Psi.

PUBLICATIONS

An Attempt to Prepare Dichloroiodoso Acetic Acid.
M. S. Thesis, University of Chicago, 1934

Potassium Guaiacol Sulfonate (with A. H. Clark).
J. Am. Pharm. Assoc., 245: 64, 1935.

Hydrolytic Cleavage and Oxidation of Soybean Meal by PENICILLIUM LUTEUM PURPOROGENUM AND ASPERGILLUS NIGER. Food research, 4:363, 1939.